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RESEARCH ARTICLE

Antimicrobial Activity of *Actinomycetes* from Soil Samples of Some Micro-Ecosystems of Satara District, Maharastra, India against Selected Human Pathogens

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ABSTRACT

In the present study, antagonistic actinomycetes from different soil samples were isolated, screened for their inhibitory activity, identified and their anti-microbial profile against different human test pathogens was determined. Three different micro-ecosystems namely, Forest area, Krishna river bank and Sugar cane farm from Satara district, Maharastra, India were chosen for the isolation of Actinomycetes from soil samples. A total of 83 actinomycetes were isolated from 45 soil samples and only five isolates (6.02%) with inhibitory activity against the sensitive strain of *Escherichia coli* (MTCC 739, IMTECH, Chandigarh) were isolated. Soil samples from the forest area, yielded greater number of antagonistic actinomycetes (10.00%) followed by the samples from Krishna river bank (7.14%) while, samples from sugarcane farm did not yield any antagonistic actinomycetes. All the five antagonistic isolates showed (100%) inhibitory activity against the test pathogen, *Shigella sonnei* and four antagonistic actinomycete isolates (80%) were inhibitory to the test pathogen, *Candida albicans*. The antagonistic actinomycete isolate FO-3 exhibited antagonism towards all the test pathogens at different levels and was subjected to chemo-taxonomical studies. Thin layer chromatography studies revealed that the isolate FO-3 belongs to cell wall chemotype-I and G:C content of genomic DNA was determined to be 70.03%.The isolate FO-3 was identified to be belonging to the genus *Streptomyces*. The outcome of the present study clearly suggests that, ecosystems rich in organic matter with a high percentage of carbon are the great sources of antagonistic actinomycetes producing novel anti-microbial compounds.

Key words: soil, antagonistic, actinomycetes, human pathogens, TLC, hyperchromicity, *Streptomyces*

INTRODUCTION

Ever since the discovery of penicillin by Alexander Fleming in 1928, diverse classes of antimicrobial compounds from various groups of living organisms both terrestrial and aquatic, are being extensively used for treating various diseases. Unfortunately, reports on the emergence of human pathogens with multiple drug-resistance are ever increasing (Spellberg *et al.,* 2004). Human pathogens which are drug resistant, pose a huge threat human medicine (Luzhetskyy *et al.,* 2007) and this has led to the exploration for novel bio-active compounds with anti-microbial properties, by the scientific community. Actinomycetes are the gram-positive, filamentous bacteria and they have been described as the greatest source of antibiotics. Diverse classes of antimicrobial compounds like, Aminoglycosides, Anthracyclines, Chloramphenicol, β-lactams, Macrolides and Tetracyclines have been isolated from this single group of bacteria and they are the sources of more than 4,000 of the naturally occurring antibiotics (Okami and Hotta, 1988). The origin of almost 80% of the world's antibiotics can be traced to actinomycetes (Pandey *et al*., 2004). Terrestrial soil samples have been a rich source of actinomycetes (Okazaki and Natio, 1986). Hence, the present study was undertaken with the objectives of isolation of actinomycetes from different soil samples, detection of inhibitory strains, determination of their antagonistic profile against selected human test pathogens and their identification using standard chemotaxonomic schemes.

MATERIALS AND METHODS

Collection of samples

Sterile polypropylene bags were used for the collection of 5-10 grams of the soil samples. Soil samples were collected aseptically from three different sampling stations namely, Forest area, Krishna river bank and Sugarcane farm from Satara district, Maharastra, India. 45 soil samples in total, with 15 samples from each of the three sampling stations, were collected aseptically and stored at refrigeration temperature till further use.

Isolation of actinomycetes

The collected soil samples were diluted using ten fold serial dilution method with sterile saline. The dilutions were thoroughly mixed with a vortex mixer for a minute and inoculation was done using spread plating onto a selective medium, Starch-Casein Agar (SCA)(Hi-Media Pvt. Ltd., Mumbai) (Table 1). SCA medium was used with two anti-fungal agents, Cycloheximide and Nystatin @ 50 μg/ml (Hi-Media Pvt. Ltd. Mumbai). Plates were incubated at room temperature for 6-7 days. The actinomycete isolates were selected based on their colony morphology with a typical chalky to leathery appearance (IMTECH, 1998) followed by gram staining, acid fast staining and subjected to light microscopy for filamentous nature, width of hyphae, nature of aerial and substrate mycelium (Cappucino and Sherman, 2004). The Gram-positive, non-acid fast isolates with aseptate hyphae were picked up and purified onto Starch Casein Agar (SCA) plates. The purified isolates were sub-cultured on SCA slants, incubated at room temperature for 6-7 days and stored at refrigeration temperature till further use.

Detection of antagonistic actinomycetes

A modified spot inoculation method of James *et al*.(1996), was employed for the primary screening of purified actinomycete isolates for their inhibitory activity, against a sensitive strain of *Escherichia coli* (MTCC 739) (IMTECH, Chandigarh). Spot inoculation of actinomycete isolates was done at the center of the Antibiotic Assay Medium (AAM) (Hi-Media Pvt. Ltd. Mumbai) (Table 2). After incubation for 6-7 days at room temperature, the plates were flooded with an overnight broth culture of the sensitive strain of *E. coli* and incubated at 37^0C for 24-48 hours. The antagonistic nature of actinomycete strains was detected by the presence of clear zones of growth inhibition of the sensitive *E. coli* strain, around their colony. Based on the extent of the zone of inhibition, the degree of antagonism of

actinomycete isolates was evaluated on a 5-point scale. Antagonistic actinomycete isolates with higher antagonistic activity were used for the secondary screening against selected human test pathogens.

Determination of Inhibitory activity of antagonistic actinomycetes

The actinomycete isolates found out to be antagonistic, were subjected to secondary screening to determine their inhibitory profile against selected Gram-negative, Gram-positive bacterial and fungal human pathogens following the modified cross-streak assay of Lemos *et al*. (1985). Nine human test pathogens, *Salmonella typhi* (MTCC 734), *Vibrio parahaemolyticus* (MTCC 451), *Salmonella paratyphi* A (MTCC 735), *Shigella flexneri* (MTCC 1457), *Shigella sonnei* (MTCC 2957), *Salmonella typhimurium* (MTCC 98), *Klebsiella pneumoniae* (MTCC 109), *Staphylococcus aureus* (MTCC 96) and *Candida albicans* (MTCC 227), procured from IMTECH ,Chandigarh were used in the assay. Inoculum from each of these inhibitory actinomycete isolates was used for making a diagonal streak onto Antibiotic Assay Medium (AAM) agar plate and incubated at room temperature for a period of 6-7 days. For streaking, the inoculation loop was straightened out and bent into L-shape so as to get a streak of 8-10 mm width. After the incubation, young culture of each of the selected human test pathogens was streaked perpendicular to the central streak of the actinomycetes culture, leaving a gap of 2 mm from the central streak. After incubation at 37^0C for 24h, the inhibitory activity of actinomycete isolates was indicated by the clear zones of growth inhibition of test pathogens near the central streak and clear zones of various test pathogens was measured in millimeters (mm). The AAM agar plates with only the test pathogens served as control.

Identification of antagonistic actinomycetes

Standard chemotaxonomic schemes of IMTECH (1998) and Goodfellow (1989) were used for the identification of actinomycete isolates with greater inhibitory activity.

Light microscopy

Actinomycete isolates with prominent antagonistic activity were sub-cultured using cover slip culture technique onto SCA medium. A novel, indigenously designed cover slip holder was used for scanning the field for the nature of aerial and substrate mycelium using a phase-contrast Nikon-make microscope (Cappucino and Sherman, 2004).

Biochemical tests and determination of cell wall chemotypes

The actinomycete isolates were subjected to casein, xanthine, urea, xylose and lactose utilization tests (Schaal, 1985). Thin Layer Chromatographic (TLC) analysis of the extracted cell wall amino acids was carried out using cellulose coated thin layer chromatography sheet, LL-Diamino Pimelic Acid (DPA), meso-DAP, DD-DAP isomer standards(Qualigens, India) and methanol: water: 6 N HCl : Pyridine (80: 26: 4: 10 v/v) as mobile phase. Visualization was carried out by spraying the plates with 0.2% (w/v) ninhydrin in acetone. The plates were heated at 105^0C for 5 minutes. Rf values of amino acids in the samples were calculated, compared with standards and identified. TLC was also carried out for the detection of characteristic sugars in the cell wall of the actinomycete isolates. Samples were run on silica gel coated TLC sheet with Glucose, Mannose, Rhamnose, Galactose, Ribose, Arabinose, Xylose as sugar standards (Qualigens, India), and acetonitrile:water (92.5:7.5 v/v) as mobile phase. The spots were visualized by spraying aniline phthalate reagent (prepared using aniline 2 ml, phthalic acid 3.3g and water saturated butanol 100 ml) and heating the plates at 100^0C for 5 minutes for visualisation. Rf values of samples were calculated, compared with standards and the sugars in the samples were identified.

Determination of the G+C content of DNA of antagonistic actinomycete isolates

The protocol of Wilson (2003) was used for the extraction of chromosomal DNA from antagonistic actinomycete isolates. For determining the temperature of melting (Tm) through hypochromicity curve, the chromosomal DNA of the isolate was heated at different increasing temperatures and the absorbance was recorded at 260 nm. The temperature was raised in increments of 2^0C from 40^0C to 96^0C and the absorbance was measured using Agilent 8453 UV-Visible Spectrophotometer with thermostat controlled cell housing and a water bath. The absorbance was recorded until its value got stabilized. The hypochromicity curve was obtained by plotting hypochromicity value (A_t /A_{25}) against temperature. Midpoint of hypochromicity curve was taken as the Tm and calculation of G+C content was done according to the formula G+C=(Tm-53.9)2.44 (Mandel & Murmur, 1968).

RESULTS

Isolation of actinomycetes

A total of 83 actinomycetes were isolated from 45 soil samples. High number of actinomycete isolates were obtained from forest area followed by Krishna river bank and Sugarcane farm (Table 3).

Detection of antagonistic actinomycetes

Out of 83 actinomycete isolates from the three sampling stations, only five isolates inhibited the growth of sensitive *E. coli* strain, constituting to 6.02% of the total number of isolates in the primary screening for the inhibitory actinomycetes (Table 4). Of the three sampling stations, the soil samples from the Forest area yielded the highest number of antagonistic actinomycetes (10.00%) followed by Krishna river bank (7.14%)(Table 4). Samples from the Sugarcane farm did not yield any of the inhibitory actinomycetes. The isolate FO-3 recorded the highest inhibitory score of 5 on a 5-point scale against the sensitive *E. coli* strain followed by FO-4 with a score of 3 and KR-2 & KR-25 each with a score of 2 (Fig 1).

Inhibitory activity of the actinomycete isolates against various test pathogens

All the five inhibitory actinomycete isolates were subjected to secondary screening to determine their inhibitory profile against selected human test pathogens. The isolate FO-3 from the Forest area exhibited prominent inhibitory activity with a zone of inhibition of ≥ 20 mm against most of the test pathogens and inhibited all the test pathogens. The isolates FO-4 and KR-25, also recorded a higher zone of inhibition of ≥ 20 mm, but against three and two of the test pathogens respectively. The isolates FO-9 and KR-2 inhibited two of the test pathogens each. The test pathogens Tp-2, *Vibrio parahaemolyticus* and Tp-6, *Shigella flexneri* did not show any growth on AAM medium and hence were not considered in the assay results(Table 5) (Fig. 2).

Identification of actinomycete isolates with antagonistic activity

All the five isolates with inhibitory activity against the sensitive strain of *E. coli* (MTCC 739) were gram positive and non-acid fast. The isolate, FO-3 which showed prominent inhibitory activity against all the test pathogens, was subjected to chemotaxonomic scheme of identification. LL-DAP and glycine were present in the cell wall hydrolysate of this isolate and hence it was classified under Cell wall chemotype-I. The whole cell sugar analysis of this isolate showed absence of characteristic sugars (Table 6) and hence was classified under the sugar pattern- C. The temperature of melting (Tm) as calculated from the hypochromicity curve was 82.6^0C (Fig 8) and the G:C content of the genomic DNA was estimated to be 70.03% (Table 6). From the above results, the isolate FO-3 was identified to be belonging to the genus *Streptomyces* (Table 6).

DISCUSSION

Isolation of actinomycetes

In the present study, high number of actinomycete isolates were obtained from Forest area followed by Krishna river bank and Sugarcane farm (Table 3). Goodfellow and Simpson (1987) reported that actinomycetes are found abundantly in cultivated and uncultivated soils in various regions throughout the world. Actinomycetes were isolated by many workers from various soil samples namely, forest soil, sugar cane field including river bank soil (Okudoh and Wallis, 2007; Bakheit and Saadabi, 2014; Patel *et al*., 2014).

Detection and Determination of antagonistic activity of actinomycetes

In the present study, samples from Forest area yielded a high percentage (10.00%) of antagonistic actinomycetes inhibitory to the sensitive *E. coli* strain (Table 4). when compared to that of Krishna river bank (7.14%) (Table 4). However, Okudoh and Wallis (2007), reported very low percentage (5.28%) of actinomycetes with inhibitory activity, isolated from forest soil samples. This may be due to the differences in the concentration of organic matter present in the soil samples of different forest areas. The reasons for high incidence of actinomycetes in general and antagonistic actinomycetes in specific, in the soil samples from Forest area in the present study, may be due to the presence of high organic load and hence, high Carbon to Nitrogen (C:N) ratio. Therefore, these soil samples favor the growth of actinomycetes as well as other bacteria also, leading to high competition between actinomycetes and other bacterial species for nutrients & space. In such micro-environments with high bacterial load, only the antagonistic actinomycetes thrive in high numbers both in terms of biodiversity as well as biomass by secreting highly diverse classes of anti-microbial compounds and inhibiting the growth of other microbes (Walker and Colwell, 1975).7.14% of inhibitory actinomycetes were isolated from soil samples of the Krishna river bank in the present study (Table 4). In contrast to the results of the present study, Okudoh and Wallis (2007) observed that none of the actinomycetes isolated from the riverside soil samples were inhibitory. In both cases, the absence or low percentage of occurrence of antagonistic actinomycetes may be due to the fact that these micro-environments might have low concentration of organic matter and hence, lesser bacterial population, leading to less competition between bacterial flora for nutrients and space. This situation might not trigger the secondary metabolite pathways in actinomycetes, responsible for the secretion of anti-microbial compounds which make the actinomycetes antagonistic.

None of the actinomycete isolates from the Sugarcane farm soil samples were found to be inhibitory, in the present study. However, Okudoh and Wallis (2007) noted in their study that 1.88% of the actinomycetes isolated from the sugarcane field were antagonistic. In both cases, the reasons for absence or very low percentage of incidence of antagonistic actinomycetes from the Sugar cane farm may be due to the application of large quantitites of inorganic fertilizers during agriculture practices, especially in sugarcane cultivation (Yadav, 2009) which has a detrimental effect on the soil microflora, leading to a significant reduction in their population (Barabasz *et al*., 2002). This might lead to very less competition for space and nutrients among the soil microbes and might not trigger the secondary metabolite pathways responsible for antagonistic activity of the actinomycetes In the present study, 6.02% of the total actinomycete isolates from different soil samples were observed to be antagonistic. However, contrary to the results of the present study, lower percentage of incidence of antagonistic actinomycetes (3.11%) were reported by Okudoh and Wallis (2007). However, in their study, Velayudham and Murugan (2012) observed a very high percentage of incidence (97.22%) of the antagonistic actinomycetes. These differences in the percentage of incidence of antagonistic actinomycetes in different studies may be attributed to the differences in concentration of organic matter leading to variations in level of competition between actinomycetes and other microbial flora for nutrients and space.

In the present study, 80.00% of the actinomycete isolates were inhibitory to the human pathogenic yeast, *Candida albicans* and 20.00% of the actinomycete isolates were antagonistic to *Staphylococcus aureus* (Table 5)(Fig. 2). However, when compared to the results of the present study, a low percentage of actinomycete isolates (55.56%) were

inhibitory to *Candida albicans* and a high percentage of actinomycete isolates (47.22%) were inhibitory to *Staphylococcus aureus* (Velayudham and Murugan, 2012). However, comparatively very low percentage of actinomycete isolates, 44.03% and 9.7%, antagonistic to *Staphylococcus aureus* and *Candida albicans*, respectively were reported by Patel *et al*.(2014). All the actinomycete isolates tested (100%) exhibited antagonism at varying levels against any one of the test pathogens in the present study (Table 5). In a study, Patel *et al.*(2014) observed that 79% of the actinomycete isolates had inhibitory activity against one or more pathogens.

Identification of antagonistic actinomycetes

The actinomycete isolate FO-3 which exhibited prominent inhibitory activity against all the test pathogens in the present study, was subjected to chemotaxonomic schemes of identification and was identified to be belonging to the genus *Streptomyces* spp. (Table 6). However, it was reported that 76% of the actinomycete isolates were identified to be belonging to the genus *Streptomyces* (Bakheit and Saadabi, 2014). In another study by Rakshanya *et al.* (2011), it was observed that 60% of the antagonistic actinomycetes isolates were belonging to *Streptomyces sp.* However, Elamvazhuthi and Subramanian (2013), reported that, 100% of their antagonistic actinomycetes were belonging to *Streptomyces sp*. Soils rich in organic matter with high carbon support higher biomass of *Strepomyces spp.* (Lee and Hwang, 2002; Bonjar, 2004). From the results of the present study it can be clearly inferred that soil samples are the great sources of actinomycetes and that actinomycetes are the greatest source of diverse anti-microbial compounds. Large number of antagonistic actinomycetes can be isolated in particular, from the soil samples of ecosystems, which are rich in organic matter with a high Carbon to Nitrogen ratio.

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Table 1: Composition of AAM (Antibiotic Assay Medium)

Media Component	Quantity (g/l)
Peptic digest of Animal tissue	6.0
Yeast extract	3.0
Beef extract	1.5
Agar	15.0
D/w	Make up to 1L
pH	7.9±0.2

Table 2: Composition of SCA (Starch Casein Agar)

Media Component	Quantity (g/l)
Soluble starch	10.0
Vitamin free casamino acids	0.3
Calcium Carbonate $CaCO_3$	0.02
Fe3SO4.7H2O	0.01
KNO3	2.0
MgSO4.7H2O	0.05
NaCl	2.0
Agar	18.0
D/w	Make upto 1L
pH	7.1±0.1

Table 3: Actinomycetes isolated from soil samples of different sampling stations

Sampling Stations	Number of Soil Samples	Number of Actinomycete Isolates
Forest Area	15	30
Krishna River Bank	15	28
Sugarcane Farm	15	25
Total	45	83

Table 4: Antagonistic Actinomycetes isolated from various soil samples of different sampling stations

Sampling Stations	Number of Actinomycete Isolates	Number of antagonistic Actinomycete Isolates
Forest Area	30	3(10.00%)
Krishna River Bank	28	2 (7.14%)
Sugarcane Farm	25	0 (0%)
Total	83	5 (6.02%)

Table 5: Zone of growth inhibition of actinomycete isolates against various human test pathogens as detected by cross-streak assay

Actinomycete Isolate	Inhibition of Test Pathogens (zone expressed in mm)								
	Tp-1	Tp-2	Tp-3	Tp-4	Tp-5	Tp-6	Tp-7	Tp-8	Tp-9
FO-3	28	NGM	30	31	28	NGM	29	18	28.5
FO-4	R	NGM	24	R	8	NGM	24	28	R
FO-9	R	NGM	R	27	R	NGM	7	R	R
KR-2	R	NGM	R	28	R	NGM	28	R	R
KR-25	R	NGM	R	25	14	NGM	24	R	7

Tp – Test Pathogen, NGM- No Growth on the Medium AAM, R- Resistant

Test Pathgoens: Tp-1, *Staphylococcus aureus*; Tp-2, *Vibrio parahaemolyticus* ; Tp-3, *Salmonella typhi*; Tp-4, *Candida albicans*; Tp-5, *Salmonella paratyphi A*; Tp-6, *Shigella flexneri*; Tp-7, *Shigella sonnei*; Tp-8, *Salmonella typhimurium*; Tp-9, *Klebsiella pneumoniae*

Table 6: Tests used for the identification of the actinomycete isolate FO-3

Test/Analysis			Result
Light Microscopy		Gram Reaction	Gram +ve
		Acid-Fast Staining	Non acid-fast
		Cellular Nature	Filamentous, Asepatate hyphae with hyphal width -0.5 - 2 µ Aerial hyphae- bear spores in spirals
Biochemical Tests		Casein decomposition	+
		Xanthine decomposition	+
		Urea decomposition	+
		Acid from Xylose	+
		Acid from Lactose	+
TLC Analysis	Cell wall amino acid	LL-DAP	Present Cell wall chemotype-I
		Meso-DAP	Absent
		DD-DAP	Absent
	Whole cell sugar pattern		Sugar pattern - C No diagnostic sugar present
G:C content of genomic DNA			70.03%

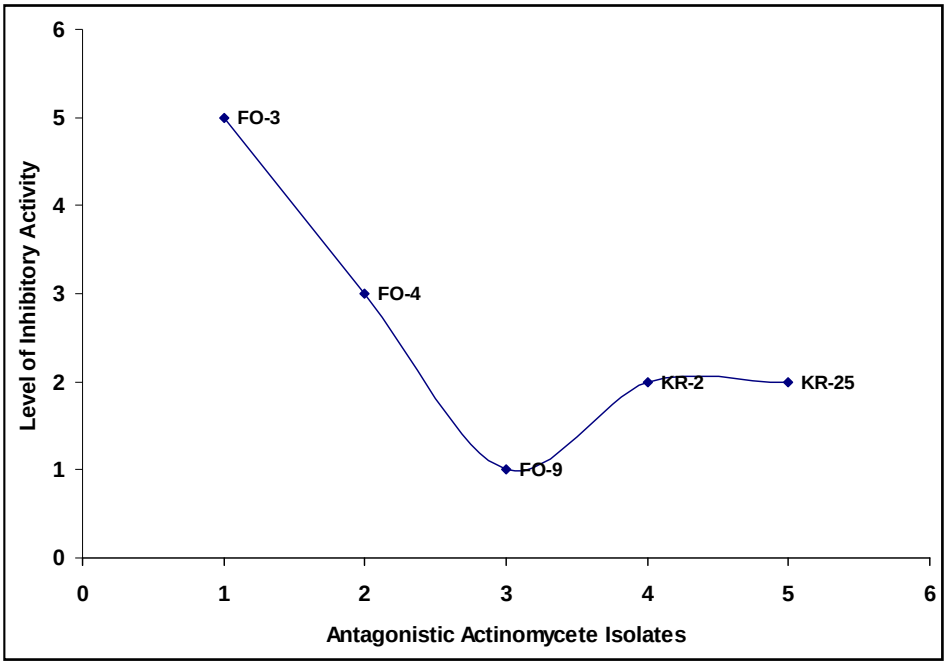

Figure 1: Inhibitory activity of actinomycete isolates against the sensitive strain of *Escherichia coli* (MTCC 739) detected and measured on a 5-point scale
5-Point Scale : 5- Highest level of inhibitory activity; 4- Higher inhibitory activity; 3- Moderate level of inhibitory activity; 2 -Lower level of inhibitory activity 1- Lowest inhibitory activity; 0- No inhibitory activity

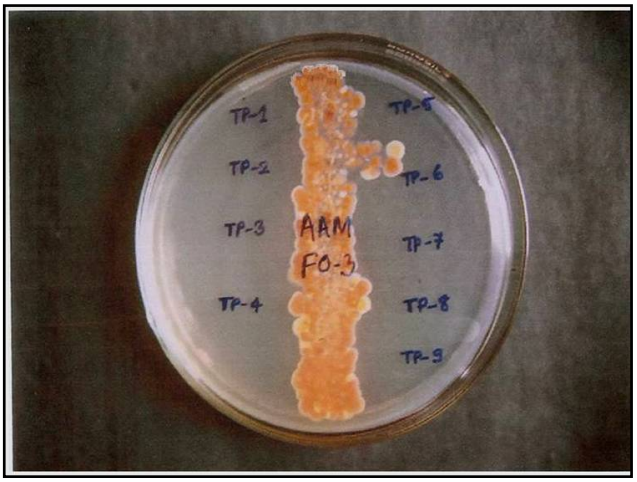

Figure 2 : Inhibitory profile of antagonistic actinomycetes against human test pathogens by cross-streak assay; Test Pathgoens: Tp-1, *Staphylococcus aureus*; Tp-2, *Vibrio parahaemolyticus* ; Tp-3, *Salmonella typhi*; Tp-4, *Candida albicans*; Tp-5, *Salmonella paratyphi A*; Tp-6, *Shigella flexneri*; Tp-7, *Shigella sonnei*; Tp-8, *Salmonella typhimurium*; Tp-9, *Klebsiella pneumonia*

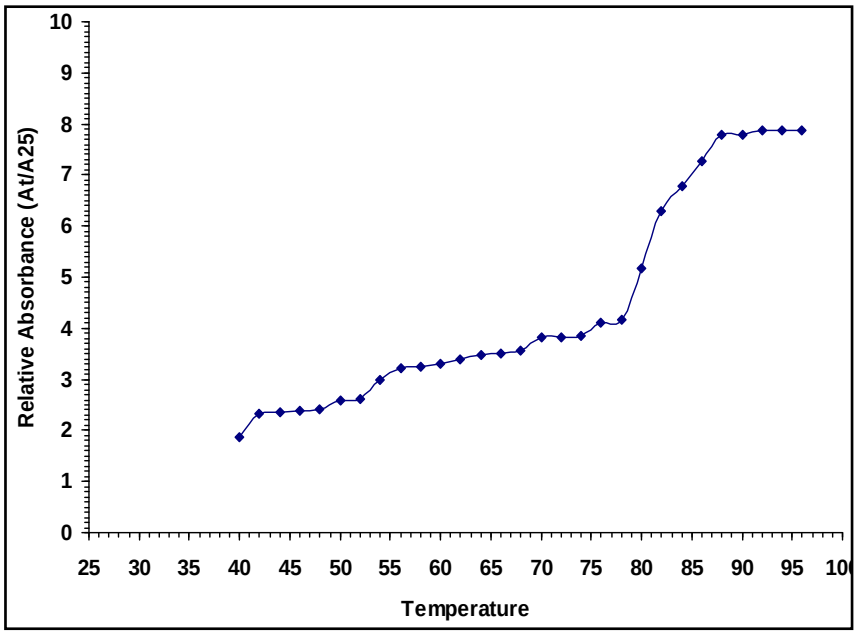

Figure 3: Hyperchromicity curve of chromosomal DNA of the antagonistic actinomycete isolate FO-3